MCH4 AND MCH5, APOPTOTIC PROTEASE, NUCLEIC ACIDS ENCODING AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a continuation of U.S. Application No.

5   09/291,692, filed April 13, 1999; which application is a continuation of pending United States Patent Application No. 08/882,429, filed June 25, 1997, now abandoned; which application is a continuation of United States Patent Application No. 08/665,220, filed June 14, 1996, now issued as United States Patent No. 5,786,173; which application is a continuation-in-part of United States Patent Application No. 08/618,408, filed

10   March 19, 1996, now issued as United States Patent No. 5,851,815; all prior applications are hereby incorporated herein by reference

STATEMENT OF GOVERNMENT INTEREST

TECHNICAL FIELD

Throughout this application various publications are referenced within parentheses. The disclosure of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of

20   the art to which this invention pertains.

BACKGROUND OF THE INVENTION

The present invention relates generally to apoptosis or, programmed cell death, and more particularly, to novel aspartate-specific cysteine proteases which can be used to modulate apoptosis for the therapeutic treatment of human diseases.

25   Apoptosis is a normal physiological process of cell death that plays a critical role in the regulation of tissue homeostasis by ensuring that the rate of new cell

accumulation produced by cell division is offset by a commensurate rate of cell loss due to death. It has now become clear that disturbances in apoptosis, also referred to as physiological cell death or programmed cell death, that prevent or delay normal; cell turnover can be just a important to the pathogenesis of diseases as are known

5   abnormalities in the regulation of proliferation and the cell cycle. Like cell division, which is controlled through complex interactions between cell cycle regulatory proteins, apoptosis is similarly regulated under normal circumstances by the interaction of gene products that either induce or inhibit cell death.

The stimuli which regulate the function of these apoptotic gene products

10   include both extracellular and intracellular signals. Either the presence or removal of a particular stimuli can be sufficient to evoke a positive or negative apoptotic signal. For example, physiological stimuli that prevent or inhibit apoptosis include, for example, growth factors, extracellular matrix, CD40 ligand, viral gene products neutral amino acids, zinc, estrogen and androgens. In contrast, stimuli which promote apoptosis

15   include growth factors such as tumor necrosis factor (TNF), Fas, and transforming growth factor β (TGFβ), neurotransmitters, growth withdrawal, loss of extracellular matrix attachment, intracellular calcium and glucocorticoids, for example. Other stimuli, including those of environmental and pathogenetic origins, also exist which can either induce or inhibit programmed cell death. Although apoptosis is mediated by

20   diverse signals and complex interactions of cellular gene products, the results of these interactions ultimately feed into a cell death pathway that is evolutionarily conserved between humans and invertebrates.

Several gene products which modulate the apoptotic process have now been identified. Although these products can in general be separated into two basic

25   categories, gene products from each category can function to either inhibit or induce programmed cell death. One family of gene products are those which are members of the Bcl-2 family of proteins. Bcl-2, is the best characterized member of this family and inhibits apoptosis when overexpressed in cells. Other members of this gene family include, for example, Bax, Bak, Bcl-x$_L$, Bcl-x$_S$, and Bad. While some of these proteins

30   can prevent apoptosis others augment apoptosis (*e.g.*, Bcl-x$_S$ and Bak, respectively.)

A second family of gene products, the aspartate-specific cysteine proteases (ASCPs), are related genetically to the *C. elegans* ced-3 gene product which was initially shown to be required for programmed cell death in the roundworm, *C. elegans*. The ASCPs family of proteases include human ICE (interleukin-1-β

5   converting enzyme), ICH-1$_L$, ICH-1$_S$, CPP32, Mch2, Mch3, ICH-2 and ICE$_{rel}$III. Among the common features of these gene products is that 1) they are cysteine proteases with specificity for substrate cleavage at Asp-x bonds, 2) they share a conserved pentapeptide sequence (QACRG; Seq. Id. No. 11) within the active site and 3) they are synthesized as proenzymes that require proteolytic cleavage at specific

10   aspartate residues for activation of protease activity. Cleavage of the proenzyme produces two polypeptide protease subunits of approximately 20kD (p20) and 10kD (p10) which, in the case of ICE, combine non-covalently to form a tetramer comprised of two p20:p10 heterodimers. Although these proteases, when expressed in cells, induce cell death, several alternative structural forms of these proteases, such as ICEδ,

15   ICEϵ, ICH-1$_S$ and Mch2β, actually function to inhibit apoptosis.

In addition to the Bcl-2 and ASCP gene families which play a role in apoptosis in mammalian cells, it has become increasingly apparent that other gene products exist which are important in mammalian cell death and which have yet to be identified. For example, in addition to Ced-3, another *C. elegans* gene known as Ced-4

20   exists which is also required for programmed cell death in *C. elegans*. However, mammalian homologies of this protein remain elusive and have not yet been identified. Further, it is ambiguous as to whether other genes exist which belong to either of the above two apoptotic gene families or what role they may play in the programmed cell death pathway. Finally, it is unclear what the physiological control mechanisms are

25   which regulate programmed cell death or how the cell death pathways interact with other physiological processes within the organism. For example, recently it has been suggested that the cytotoxic T-lymphocytes mediate their destructive function by inducing apoptosis in their target cells.

Apoptosis functions in maintaining tissue homeostasis in a range of

30   physiological processes such as embryonic development, immune cell regulation and

normal cell turnover. Therefore, the dysfunction, or loss of regulated apoptosis can lead to a variety of pathological disease states. For example, the loss of apoptosis can lead to the pathological accumulation of self-reactive lymphocytes such as that occurring with many autoimmune diseases. Inappropriate loss of apoptosis can also lead to the

5   accumulation of virally infected cells and of hyperproliferative cells such as neoplastic or tumor cells. Similarly, the inappropriate activation of apoptosis can also contribute to a variety of pathological disease states including, for example, acquired immunodeficiency syndrome (AIDS), neurodegenerative diseases and ischemic injury. Treatments which are specifically designed to modulate the apoptotic pathways in these

10   and other pathological conditions can change the natural progression of many of these diseases.

Thus, there exists a need to identify new apoptosis genes and their gene products and for methods of modulating this process for the therapeutic treatment of human diseases. The present invention satisfied this need and provides related

15   advantages as well.

SUMMARY OF THE INVENTION

The invention provides an isolated gene encoding Mch4 or an isolated gene encoding Mch5 as well as functional fragments thereof. Also provided are isolated nucleic acid sequences encoding Mch4 or Mch5 or functional fragment thereof. The

20   gene or nucleic acid sequences can be single or double stranded nucleic acids corresponding to coding or non-coding strands of the Mch4 or Mch5 nucleotide sequences. Also provided are genes and nucleic acids encoding functional fragments such as the FADD-like domains Mch4A, Mch4B, or Mch5A and Mch5B. Isolated Mch4 or Mch5 polypeptides or functional fragments thereof including the FADD-like

25   domains Mch4A, Mch4B, Mch5A and Mch5B are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide and predicted amino acid sequence of Mch4 (SEQ ID NOS:1 and 2, respectively).

Figure 2 shows the nucleotide and predicted amino acid sequences of Mch5 (SEQ ID NOS:3 and 4, respectively).

Figure 3 shows the amino acid sequences of Mch4 and Mch5 and their homology to other ASCP sequences. (A) Colinear alignment of human FADD (SEQ ID NO:64) with the two FADD-like domains in both Mchk4 and Mch5. These domains have been denoted as Mch4A (amino acid residues 18-105; SEQ ID NO:65), Mch4B (amino acid 112-189; SEQ ID NO:66), Mch5A (amino acid 4-77; SEQ ID NO:67) and Mch5B (amino acid 128-205; SEQ ID NO:68). The first domain of Mch4 (Mch4A) has the highest homology to the N-terminal 79 amino acid long FADD (gb accession #U24231) death effector domain (37% identity, 57% similarity) than the second domain (Mch4B) 22% identity, 53% similarity). Mch4A also shows high homology to PEA-15 (gb accession #X86694) and KIAA0179 (gb accession #D80001) proteins. Mch4B shows some homology to SRB7 (gb accession #U46837) and yeast cdc4 (gb accession #Z45255) proteins. (B) Multiple sequence alignment of all known human ASCPs and the nematode Ced-3 ASCP. The active site pentapeptide QACRG/QACQG (SEQ ID NOs: 11 and 10 respectively) is boxed. Based on crystal structure of ICE, the numbered residues within the ICE sequence are involved in catalysis (open boxes), and binding the substrate-carboxylate of P1 Asp (open circles). The residue adjacent to the substrate P2-P4 amino acids are indicated by closed triangles. D/X indicates known and potential processing sites between the small and large subunits of ASCPs. The Roman numbers on the right indicate the three ASCP-subfamilies; the Ced-like subfamily (I), the ICE-like subfamily (II) and the NEdd2/Ich-1 subfamily (III). The asterisk indicates the nonconservative Arg to Gln substitution in Mch4 and Mch5.

Figure 4 shows the cleavage of CPP32 proenzyme by Mch4 and granzyme B. (A) Effect of Asp175 mutation on cleavage of proCPP32. ^{35}S-labeled wild type proCPP32 (Mut-D175, -lanes) or Asp175-mutated proCPP32 (Mut-D175, + lanes) were incubated with recombinant Mch4 (Mch4, +lanes), granzyme B (GraB, +lanes) or buffer (Mch4 and GraB, -lanes) for 1 h at 37°C. The reaction products were then analyzed by SDS-PAGE and autoradiography. (B) Effect of Asp9 mutation and the DEVD-CHO inhibitor on cleavage of the propeptide of proCPP32. 35S-labeled wild

type proCPP32 (mut-D9 and Mut-175, -lanes) or Asp9-mutated (mut-D9, +lanes) or Asp 175-mutated (Mut-D175, +lanes) proCPP32 were incubated with granzyme B (GraB, +lines) or buffer (GraB, -lanes) in the presence (+lanes) or absence (-lanes) of the DEVD-CHO inhibitor. The reaction products were analyzed as above. SS, indicates the small subunit. LS, indicates the large subunit.

Figure 5 shows Cleavage of Mch3 and Mch4 proenzymes by Mch4 and granzyme B. (A) Effect of Aspartate mutations on cleavage of proMch3 (A) or Mch4 (B). ^{35}S-labeled wild type proMch3 (WITH THE lanes), Asp198-mutated proMch3 (M lanes), truncated Mch4-M134 (T1 lanes), truncated Mch4-M235 (T2 lanes) or Asp372-mutated Mch4-M134 (MT lanes) were incubated with recombinant Mch4 (Mch4, + lanes), granzyme B (GraB, + lanes) or buffer (Mch4 and GraB, -lanes) for 1 hour at 37°C. The reaction products were then analyzed by SDS-PAGE and autoradiography. SS, indicates the small subunit. LS, indicates the large subunit.

Figure 6 shows CPP32 and Mch3 activity towards Mch4 proenzyme. 35S-labeled wild type Mch4 (Mut, -lanes) or Asp239-mutated Mch4 (Mut, +lanes) were incubated with recombinant CPP32 (CPP32, + lanes), Mch3 (Mch3, + lanes) or buffer (CPP32 and Mch3, -lanes) for 1 h at 37°C. The reaction products were then analyzed by SDS-PAGE and autoradiography.

Figure 7 shows potential apoptotic protease cascades involving the activation of multiple ASCP family members.

Figure 8 shows MCF7 cells co-transfected with either plasmid pcDNA3 or Mch5B and pCMV-SPORT-βgal. Following 24 hours post-transfection, cells were fixed and stained with X-gal. Percentage of blue cells that were non-apoptotic is shown (i.e., viable cells). Non-apoptotic cells were distinguished from apoptotic cells by their flattened, spread morphology in the microscope using phase contrast optics (as opposed to rounded, pynotic or apoptotic morphology).

DETAILED DESCRIPTION OF THE INVENTION

This invention is directed to novel cell death specific proteases termed Mch4 and Mch5. These proteases are members of the aspartate-specific cysteine

protease (ASCP) family of proteases which includes, for example, ICE, ICH-1$_L$, ICH-1$_S$, CPP32, Mch2, Mch3, ICH-2 and ICE$_{rel}$III. Similar to other ASCPS, Mch4, and Mch5 are synthesized as a larger proenzyme and become active following proteolytic cleavage into two subunits; large subunit of approximately 17-27 kD and small subunit of

5   approximately 10-12 kD. The two subunits form heterodimers which associate with each other into an active complex. Substrate specificity uniquely requires an Asp residue in the P1 position of the substrate binding site with a small, preferably hydrophobic residue in the P1' position. In addition, the N-terminus of both Mch4 and Mch5 contain FADD-like death effector domains indicating their interaction with

10   FADD. This interaction further indicates that through these FADD-like domains, Mch4 and Mch5 function in the fas mediated apoptotic pathway.

In one embodiment, the invention is directed to nucleic acids encoding the apoptotic cysteine protease Mch4 or Mch5. The nucleic acids are used to produce recombinant Mch4 or Mch5 proteases, whose activity can be measured emzymatically.

15   The recombinant polypeptides are used to screen for Mch4 or Mch5 inhibitory compounds. Mch4 or Mch5 inhibiting compounds include those which inhibit protease activity as well as compounds which inhibit Mch4 or Mch5 binding to other polypeptides through FADD-like domains. Such pharmaceutical compounds are useful for the treatment or prevention of diseases which are characterized by apoptotic cell

20   death. Alternatively, the Mch4 or Mch5 polypeptides can be used to screen for pharmaceutical compounds which activate or act as agonists of Mch4 or Mch5 such as by inducing cleavage of the proenzyme into its active subunits or altering polypeptide interactions through their FADD-like domains. Such compounds are useful for the treatment or prevention of diseases which are characterized by the loss of apoptotic cell

25   death.

As used herein, the term "substantially" when referring to Mch4 or Mch5 nucleotide or amino acid sequence is intended to refer to the degree to which two sequences of between about 15-30 or more nucleotides in length, are identical or similar so as to be considered by those skilled in the art to be functionally equivalent. For

30   example, the Mch4 or Mch5 nucleic acids of the invention have a nucleotide sequence

substantially the same as that shown in Figures 1 and 2 and as SEQ ID NOS:1 and 3, respectively. Thus, if a second sequence is substantially the same as that shown in SEQ ID NOS:1 and 3, then it is considered functionally equivalent by those skilled in the art. Methods for sequence comparisons and determinations of similarity are well known and routine within the art.

Functionally equivalent nucleic acid sequences include, for example, sequences that are related, but different and encode the same Mch4 or Mch5 polypeptide due to the degeneracy of the genetic code as well as sequences that are related, but different and encode a different Mch4 or Mch5 polypeptide that exhibits similar functional activity. In both cases, the nucleic acids encode functionally equivalent gene products. Functional fragments of Mch4 or Mch5 encoding nucleic acids such as oligonucleotides, polyoligonucleotides, primers and the like are also considered to be within the definition of the term and the invention as claimed. Functional equivalency is also relevant to Mch4 or Mch5 nucleic acids which do not encode gene products, for example, but instead are functional elements in and of themselves. Specific examples of such functional nucleic acids include, for example, promoters, enhancers and other gene expression regulatory elements.

Mch4 or Mch5 polypeptides of the invention have an amino acid sequence substantially similar to that shown in Figures 1, 2, and 3 and in SEQ ID NOS:2 and 4, respectively. Functionally equivalent Mch4 amino acid sequences similarly includes, for example, related, but different sequences so long as the different polypeptide exhibits at least one functional activity of Mch4 or Mch5. Such related, but different polypeptide include, for example, substitutions of conserved and non-essential amino acids. Fragments and functional domains of Mch4 or Mch5 are similarly included within the definition of the term and the claimed invention.

Therefore, it is understood that limited modifications may be made without destroying the biological function of the Mch4 or Mch5 polypeptide and that only a portion of the entire primary structure may be required in order to effect activity. For example, minor modifications of the Mch4 or Mch5 amino acid sequences (SEQ ID NOS:2 and 4) which do not destroy their activity also fall within the definition of Mch4

or Mch5 and within the definition of the polypeptide claimed as such. Also, for example, genetically engineered fragments of Mch4 or Mch5 either alone or fused to heterologous proteins such as fusion proteins that retain measurable enzymatic or other biological activity fall within the definition of the polypeptide claimed as such.

5   It is understood that minor modifications of primary amino acid sequence may result in polypeptides which have substantially equivalent or enhanced function as compared to the sequences set forth in Figures 1 and 2 (SEQ ID NOS 2 and 4). These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental such as through mutation in hosts which are Mch4 or Mch5 producers.

10 All of these modifications are included as long as Mch4 or Mch5 biological function is retained. Further, various molecules can be attached to Mch4 or Mch5, for example, other proteins, carbohydrates, lipids, or chemical moieties. Such modification are included within the definition of Mch4 or Mch5 polypeptides.

The invention provides a gene encoding Mch4 or Mch5, or fragment thereof. The invention also provides an isolated nucleic acid sequence encoding Mch4

15 or Mch5, or fragment thereof. The gene and nucleic acid sequences encode substantially the sequence as shown in SEQ ID NOS:1 and 3. Fragments of the gene or nucleic acid sequence are provided which comprise single or double stranded nucleic acids having substantially the sequences shown in SEQ ID NOS:1 and 3.

20   The Mch4 or Mch5 nucleic acids of the present invention were identified and isolated by a novel approach of searching a human database of expressed sequence tags (ESTs) under various stringencies to identify potential new sequence fragments which may have homology to the ICE family of cysteine proteases. As described below, such a search identified the Mch4 and Mch5 nucleic acids of the present

25 invention and also resulted in the reclassification of the cell death protease family. Previously these proteases were referred to as the ICE-family of proteases and thus the initial search criteria was directed to "ICE family" of cell death proteases. However, with the identification of Mch4 and Mch5, the proteases can now be divided into three subfamilies referred to herein as the Ced-like, ICE-like and Nedd2/ICH-1-like

30 subfamilies of cell death proteases (see Figure 3B).

In regard to the search for potential new sequences having homology to the previously referred to ICE family of proteases, novel sequences identified from the search as having homology to the ICE family of cell death proteases are then used to design primers for attempting PCR amplification and cloning of the actual cDNA. The second primer for the amplification is designed to encompass homologous regions in nucleic acid sequences that encode know ICE protease family members. In this specific case, the primer was directed to the GSWFI/GSWYI (SEQ ID NOs: 69 and 70 respectively) pentapeptide sequence that is conserved in a number of the ICE/Ced-3 family of proteases. The primer designs should take into account the predicted strandedness of both the EST sequence primer and the known primer. Thus, only if the homology search and primer hybridization conditions are successfully determined, will such an approach allow PCR amplification of a fragment of the putative novel protease cDNA.

As searching a genetic data base will yield homologous sequence matches to any query nucleotide sequence, additional criteria must be used to identify the authentic ICE subfamily homologue from among the non-specific homology matches. ICE family members share the highest degree of homology in the active site and catalytically important amino acid residues. A given EST returned by the search may not include one of these highly homologous sites, but rather, may only include a region within the protease with cryptic homology. Confirming an EST as a novel ICE protease involves translation of all the positive EST hits in three different reading frames and subsequent identification of conservative active site or catalytically important amino acid sequence motifs. Then, using conventional cDNA cloning, a full length cDNA of the putative novel protease can be obtained and 1) analyzed for overall structural homology to ICE family members, 2) recombinantly expressed and analyzed for cysteine protease activity, and 3) analyzed for the induction of programmed cell death by heterologous expression of the cDNA in appropriate cells.

Alternative methods than that described above for isolating Mch4 or Mch5 encoding nucleic acids can similarly be employed. For example, using the teachings described herein, those skilled in the art can routinely isolate and manipulate

Mch4 or Mch5 nucleic acids using methods well known in the art. All that is necessary

is the sequence of the Mch4 or Mch5 encoding nucleic acids (Figures 1 and 2 and SEQ

ID NOS:1 and 3) or their amino acids sequences (Figures 1 and 2 and SEQ ID NOS:2

and 4). Such methods include, for example, screening a cDNA or genomic library by

5   using synthetic oligonucleotides, nucleotides, nucleic acid fragments or primers as

hybridization probes. Alternatively, antibodies to the Mch4 or Mch5 amino acid

sequence or fragments thereof can be generated and used to screen an expression library

to isolate Mch4 or Mch5 encoding nucleic acids. Other binding reagents to Mch4 or

Mch5 polypeptides can similarly be used to isolate Mch4 or Mch5 polypeptides having

10   substantially the amino acid sequence show in Figure 1 and 2. Similarly, substrate

reagents such as non-cleavable peptide analogues of cysteine proteases and FADD-like

domain binding polypeptides can be used to screen and isolate Mch4 or Mch5

polypeptides.

In addition, recombinant DNA methods currently used by those skilled in

15   the art include the polymerase chain reaction (PCR) which, combined with the Mch4 or

Mch5 nucleotide and amino acid sequence described herein, allows reproduction of

Mch4 or Mch5 encoding sequences. Desired sequences can be amplified exponentially

starting from as little as a single gene copy by means of PCR. The PCR technology is

the subject matter of United States Patent Nos. 4,683,195, 4,800,159, 4,754,065, and

20   4,683,202 all of which are incorporated by reference herein.

The above described methods are known to those skilled in the art and

are described, for example, in Sambrook et al., Molecular Cloning: A Laboratory

Manual, Cold Spring Harbor Laboratory, New York (1992) and the various references

cited therein and in Ansubel et al., Current Protocols in Molecular Biology, John Wiley

25   and Sons, Baltimore, MD (1989); and in Harlow et al., Antibodies: A Laboratory

Manual, Cold Spring Harbor, New York (1989). These references and the publications

cited therein are hereby expressly incorporated herein by reference.

The invention provides an isolated Mch4 or Mch5 polypeptides

comprising substantially the amino acid sequence as that shown in Figures 1 and 2

30   (SEQ ID NOS:2 and 4). Mch4 or Mch5 functional fragments are also provided.

Specific examples of Mch4 or Mch5 functional fragment are, for example, the catalytic domain which contain the active site amino acid sequence QACQG (SEQ ID NO:10) and the FADD-like domains Mch4A, Mch4B, Mch5A or Mch5B. When compared to the active site amino acid sequence of other ASCP family members, QACRG (SEQ ID

5   NO:11), this active site sequence is similar but differs at position 4 with R substituted by Q.

Isolated Mch4 or Mch5 polypeptides of the invention can be obtained by a variety of methods known within the art. For example, the isolated peptides can be purified by biochemical methods including, for example, affinity chromatography.

10   Affinity matrices which can be used for Mch4 or Mch5 isolation can be anti-Mch4 or anti-Mch5 monoclonal or polyclonal antibodies prepared against the sequence shown in Figures 1 and 2 (SEQ ID NOS:2 and 4), or fragments thereof such as synthetic peptides. Additionally, FADD-like domain binding polypeptides which are capable of binding the FADD-like domains at the N-terminus of Mch4 or Mch5 can also be used as affinity

15   matrices. Alternatively, substrate analogues or enzymatic inhibitors of Mch4 or Mch5 can similarly be used as affinity matrices to isolate substantially pure Mch4 or Mch5 polypeptides of the invention.

Mch4 or Mch5 polypeptides can also be produced by recombinant methods known to those skilled in the art. Recombinant Mch4 or Mch5 polypeptides

20   include, for example, an amino acid sequence substantially the same as that shown in Figures 1 and 2 (SEQ ID NOS:2 and 4) as well as fusion proteins and fragments thereof. The Mch4 or Mch5 encoding nucleic acids can be cloned into the appropriate vectors for propagation, manipulation and expression. Such vectors are known or can be construed by those skilled in the art and should contain all expression elements

25   necessary for the transcription, translation, regulation, and if desired, sorting of the Mch4 or Mch5 polypeptides. The vectors can also be for use in either procaryotic or eucaryotic host systems so long as the expression and regulatory elements are of compatible origin. One of ordinary skill in the art will know which host systems are compatible with a particular vector. The recombinant polypeptides produced can be

30   isolated by the methods described above.

Apoptosis plays a significant role in numerous pathological conditions in that programmed cell death is either inhibited, resulting in increased cell survival, or enhanced which results in the loss of cell viability. Examples of pathological conditions resulting from increased cell survival include cancers such as lymphomas, carcinomas

5 and hormone dependent tumors. Such hormone dependent tumors include, for example, breast, prostate and ovarian cancer. Autoimmune diseases such as systemic lupus erythematosus and immune-mediated glomerulonephritis as viral infections such as herpesvirus, poxyvirus and adenovirus also result from increased cell survival or the inhibition of apoptosis.

10 In contrast, apoptotic diseases where enhanced programmed cell death is a prevalent cause generally includes, for example, degenerative disorders such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Retinitis pigmentosa, and Cerebellar degeneration. Other diseases associated with increased apoptosis include, for example, myelodysplastic syndromes such as aplastic anemia and

15 ischemic injury including myocaridal infarction, stroke and reperfusion injury.

The Mch4 or Mch5 encoding nucleic acids and polypeptides of the invention can be used to diagnose, treat or reduce the severity of cell death mediated diseases such as those described above as well as other diseases mediated by either increased or decreased programmed cell death. Additionally, the Mch4 or Mch5

20 encoding nucleic acids and polypeptides of the invention can be used to screen for pharmaceutical compounds and macromolecules which inhibit or promote Mch4 or Mch5 mediated apoptosis.

For example, the Mch4 or Mch5 encoding nucleic acids, polypeptides and functional fragments thereof can be used to diagnose, or to generate reagents to

25 diagnose diseases mediated or characterized by programmed cell death. Diagnosis can be by nucleic acid probe hybridization with Mch4 or Mch5 containing nucleotide sequences, antibody or ligand mediated detection with Mch4 or Mch5 binding agents or by enzyme catalysis of detectable Mch4 or Mch5 substrates. Such methods are routine to those skilled in the art. Detection can be performed *ex vivo*, for example, by

30 removing a cell or tissue sample from an individual exhibiting or suspected of

exhibiting a cell death mediated disease. Correlation of increased Mch4 or Mch5 expression or activity is indicative of diseases characterized by enhanced programmed cell death whereas correlation of decreased Mch4 or Mch5 expression or activity is indicative of diseases characterized by the inhibition of programmed cell death.

5   The above Mch4 or Mch5 polypeptides can also be formulated into pharmaceutical compositions known within the art for the treatment of cell death mediated diseases characterized by increased cell survival and proliferation. Functional fragments and peptides such as the FADD-like domains and the catalytic domain of Mch4 or Mch5 can similarly be formulated for the treatment of such diseases associated

10   with increased cell survival and proliferation. Additionally, molecules which interact with Mch4 and Mch5 can additionally be used to induce Mch4 and Mch5 mediated apoptosis. Such molecules can include, for example, FADD and FADD or fas-activators. Administration of Mch4 or Mch5 polypeptides and functional fragments thereof will induce apoptosis in treated cells and eliminate those cells characterized by

15   increased cell survival or proliferation. Administration of non-Mch4 or Mch5 polypeptides that do not directly act on Mch4 or Mch5 substrates but induce the activation of the Mch4 or Mch5 protease can similarly be used for the treatment of diseases characterized by increased cell survival and proliferation.

To be effective, the Mch4 or Mch5 polypeptides must be introduced into

20   the cells characterized by increased cell survival. Introduction can be accomplished by a variety of means known within the art including, for example, lipid vesicles and receptor mediated endocytosis. Targeting to the appropriate cell type can similarly be accomplished through conjugation to specific receptor ligands, specific target cell antibodies and the like.

25   The Mch4 or Mch5 polypeptides are administered by conventional methods, in dosages which are sufficient to induce apoptosis in the cells characterized by increased cell survival or proliferation. Such dosages are known or can be easily determined by those skilled in the art. Administration can be accomplished by, for example, intravenous, interperitonal or subcutaneous injection. Administration can be

30   performed in a variety of different regimes which include single high dose

administration or repeated small dose administration or a combination of both. The dosing will depend on the cell type, progression of the disease and overall health of the individual and will be known or can be determined by those skilled in the art.

In contrast to the induction of Mch4 or Mch5 mediated apoptosis for the
5   treatment of pathological conditions characterized by increased cell survival or proliferation, inhibitors of Mch4 or Mch5 can be used to treat diseases characterized by increased programmed cell death. Such inhibitors can be, for example, inhibitors of the Mch4 or Mch5 protease activity or inhibitors of the conversion of the inactive, pro-Mch4 or pro-Mch5 into the active Mch4 and Mch5 proteases, or alternatively inhibitors
10   of the binding activity of the FADD-like domains. Specific examples of such inhibitors can include, for example, anti-Mch4 or anti-Mch5 antibodies, proteins, or small peptidyl protease inhibitors, or small non-peptide, organic molecule inhibitors which are formulated in a medium which allows introduction into the desired cell type. Alternatively, such inhibitors can be attached to targeting ligands for introduction by
15   cell mediated endocytosis and other receptor mediated events. Specific examples of Mch4 or Mch5 peptidyl inhibitors are described in Table I of Example III and includes suicide inhibitors and substrate analogues such as the tetrapeptide DEVD aldehyde (SEQ ID NO:71) and the cowpox virus protein Crm A, for example.

Other inhibitors of Mch4 or Mch5 include, for example, small molecules
20   and organic compounds which bind and inactivate Mch4 or Mch5 by a competitive or non-competitive type mechanism. Molecules or compounds which indirectly inhibit the Mch4 or Mch5 pathway can also be used as inhibitors of Mch4. Mch4 or Mch5 inhibitors can be identified by screening for molecules which demonstrate specific or beneficial Mch4 or Mch5 inhibitory activity. Such methods are described further below
25   and can be practiced by those skilled in the art given the Mch4 or Mch5 nucleotide and amino acid sequences described herein.

Dominant/negative inhibitors of Mch4 or Mch5 can also be used to treat or reduce the severity of diseases characterized by increased programmed cell death. In this regard, Mch4 or Mch5 large subunits which lack the active site QACQG (SEQ ID
30   NO:10) can be used to bind the small subunits of Mch4 or Mch5 and prevent active

protease complexes from forming. Such a mechanism of dominant negative inhibition of Mch4 is similar to the dominant negative inhibition of Ich-1$_L$ by Ich-1$_S$. Subunits from other ASCPs can similarly be used as dominant/negative inhibitors of Mch4 or Mch5 activity and therefore treat diseases mediated by programmed cell death. Such subunits should be selected so that they bind either the p17 or p12 Mch4 or Mch5 polypeptides and prevent their assembly into active tetrameric protease complexes. Moreover, Mch4 or Mch5 subunits which have been modified so as to be catalytically inactive can also be used as dominant negative inhibitors of Mch4. Such modifications include, for example, mutation of the active site cysteine residue to include but not limited to Alanine or glycine.

Mch4 or Mch5 substrate antagonists can similarly be used to treat or reduce the severity of diseases mediated by increased programmed cell death. Such substrate antagonists can bind to and inhibit cleavage by Mch4. Inhibition of substrate cleavage prevents commitment progression of programmed cell death. Substrate antagonists include, for example, ligands and small molecule compounds.

Treatment or reduction of the severity of cell death mediated diseases can also be accomplished by introducing expressible nucleic acids encoding Mch4 or Mch5 polypeptides or functional fragments thereof into cells characterized by such diseases. For example, elevated synthesis rates of Mch4 or Mch5 can be achieved by, for example, using recombinant expression vectors and gene transfer technology. Similarly, treatment or reduction of the severity of cell death mediated diseases can also be accomplished by introducing and expressing antisense Mch4 or Mch5 nucleic acids so as to inhibit the synthesis rates of Mch4 or Mch5. Such methods are well known within the art and will be described below with reference to recombinant viral vectors. Other vectors compatible with the appropriate targeted cell can accomplish the same goal and therefore can be substituted in the methods described herein in place of recombinant viral vectors.

Recombinant viral vectors are useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the lifecycle of retroviruses and is the

process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is a large area becomes rapidly infected, most of which were not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter

5   progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

Typically, viruses infect and propagate in specific cell types. Therefore, the targeting specificity of viral vectors utilizes this natural specificity to in turn

10   specifically introduce a desired gene into predetermined cell types. The vector to be used in the methods of the invention will depend on desired cell type to be targeted. For example, if neurodegenerative diseases are to be treated by decreasing the Mch4 or Mch5 activity of affected neuronal cells then a vector specific for cells of the neuronal cell lineage should be used. Likewise, if diseases or pathological conditions of the

15   hematopoietic system are to be treated, than a viral vector that is specific for blood cells and their precursors, preferably for the specific type of hematopoietic cell, should be used. Moreover, such vectors can additionally be modified with specific receptors or ligands and the like to modify or alter target specificity through receptor mediated events. These modification procedures can be performed by, for example, recombinant

20   DNA techniques or synthetic chemistry procedures. The specific type of vector will depend upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well known methodology.

Viral vectors encoding Mch4 or Mch5 nucleic acids or inhibitors of

25   Mch4 or Mch5 such as antisense nucleic acids can be administered in several ways to obtain expression of such sequences and therefore either increase or decrease the activity of Mch4 or Mch5 in the cells affected by the disease or pathological condition. If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site.

30   However, local administration can provide a quicker and more effective treatment.

Administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into the spinal fluid can also be used as a mode of administration, especially in the case of neurodegenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with

5   the appropriate target specificity for infection.

As described above, one mode of administration of Mch4 or Mch5 encoding vectors can be by direct inoculation locally at the site of the disease or pathological condition. Local administration is advantageous because there is no dilution effect and therefore a smaller dose is required to achieve Mch4 or Mch5

10   expression in a majority of the targeted cells. Additionally, local inoculation can alleviate the targeting requirement required with other forms of administration since a vector can be used that infects all cells in the inoculated area. If expression is desired in only a specific subset of cells within the inoculated area then promoter and expression elements that are specific for the desired subset can be used to accomplish this goal.

15   Such non-targeting vectors can be, for example, viral vectors, viral genomes, plasmids, phagemids and the like. Transfection vehicles such as liposomes can be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art. Alternatively, however, nontargeting vectors can be administered directly into a tissue of any

20   individual. Such methods are known within the art and are described by, for example, Wolff et al. (Science 247:1465-1468 (1990)).

Additional features can be added to the vectors to ensure safety and/or enhance therapeutic efficacy. Such features include, for example, markers that can be used to negatively select against cells infected with the) recombinant virus. An

25   example of such a negative selection marker is the TK gene described above that confers sensitivity to the antibiotic gancyclovir. Negative selection is therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce mutant forms of Mch4 or Mch5, dysfunction of apoptosis will not occur.

As described previously, the Mch4 or Mch5 encoding nucleic acids and Mch4 or Mch5 polypeptides of the invention can be used to screen for compounds which inhibit or enhance the expression of Mch4 or Mch5 mediated apoptotic activity. Mch4 or Mch5 mediated apoptotic activity includes, for example, both the protease

5   activity of these ASCPs and/or the FADD-like domain binding activity. Such screening methods are known to those skilled in the art and can be performed by either *in vitro* or *in vivo* procedures. For example, described in Example II is a specific *in vitro* assay for Mch4 or Mch5 protease activity. This assay employs Mch4 or Mch5 polypeptide expressed in an active, processed form recombinantly in *E. coli*, whose protease activity

10   is measured by incubation with a fluorescent substrate (DEVD-AMC; Seq. Id No. 71). Also described therein are peptide and polypeptide inhibitors of Mch4. This assay can be used to screen synthetic or naturally occurring compound libraries, including macromolecules, for agents which either inhibit or enhance Mch4 or Mch5 activity. The Mch4 or Mch5 polypeptides to be used in the assay can be obtained by, for

15   example, *in vitro* translation, recombinant expression or biochemical procedures. Methods other than that described in Example II can also be used to screen and identify compounds which inhibit Mch4 or Mch5. Such methods can include, for example, binding assays such as ELISA and RIAs using FADD-like domain binding proteins. A specific example is phage display peptide libraries where greater than 10^8 peptide

20   sequences can be screened in a single round of panning. Such methods as well as others are known within the art and can be utilized to identify compounds which inhibit or enhance Mch4 or Mch5 activity.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also included within the

25   definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

CLONING AND CHARACTERIZATION OF MCH4

This Example shows the cloning, sequence analysis and tissue distribution of Mch4 and Mch5. The results described herein indicate that Mch4 and Mch5 are novel members of the cell death family of aspartate-specific cysteine proteases.

To identify potentially novel members of the ICE family of cysteine proteases, an approach combining information from the GenBank database of human expressed sequence tags (ESTs) and PCR was employed. Initially, Ced-3/ICE-like apoptotic cysteine proteases from Jurkat T-lymphocytes were enriched by amplification of a human Jurkat cDNA library using degenerate PCR primers encoding the conserved GSWFI/GSWYI (SEQ ID NOs: 69 and 70 respectively) pentapeptides (Fernandes-Alnermi et al., Cancer Res. 55:2737-2742 (1995a)). This amino acid sequence has been found to be conserved among ICE family members. Briefly, a 10 μl aliquot of human Jurkat λ Uni-Zap™ XR cDNA library containing approximately 10^8 pfu was denatured at 99°C for 5 min. and used as a substrate for PCR amplification with a degenerate primer encoding the pentapeptide GSWFI/GSWYI (SEQ ID NOs: 69 and 70 respectively) and a T3 vector-specific primer (Stratagene).

The enriched library was then amplified with a primer derived from an EST sequence identified in a homology search of the GenBank database using a query nucleotide sequence corresponding to the Mch2 and CPP32 coding sequence. The secondary amplification was performed starting with a 10 μl aliquot of the above amplified sequences combined with a primer derived from the GenBank sequence T96912 (primer T96-prl: TCAGCCTCGGCAGGAATAC SEQ ID NO:5) and a second vector specific primer (SK-Zap: CAGGAATTCGGCACGAG, SEQ ID NO:6). The secondary amplification products were cloned into a Sma I cut pBluescript II KS+ vector. All clones were screened by PCR using a degenerate oligonucleotide corresponding to the conserved active site amino acid sequence QACRG (SEQ ID NO:11) and the SK-Zap primer. Clones that were positive for the presence of the QACRG (SEQ ID NO:11) coding sequence were then subjected to DNA sequencing

using T3 and T7 sequencing primers (Stratagene). This amplification and screen resulted in the identification of a Ced-3/ICE-like partial cDNA with high homology to CPP32 and Ced-3.

Partial cDNA identified from the QACRG (SEQ ID NO:11) screening
5 was then excised from the vector, radiolabeled and used to screen the original Jurkat λ Uni-Zap™ XR cDNA library for full length cDNA clones. Positive λ clones were purified, rescued into the pBluescript II SK⁻ plasmid vector and sequenced.

The above screening identified a 3.6 kb cDNA clone from the human Jurkat T-lymphocyte cDNA library. This cDNA contains an open reading frame of
10 1437 bp that encodes a 479-amino acid protein, named Mch4 (SEQ ID NOS:1 and 2, respectively). As shown in Figures 1 and 3A, proMch 4 is a polypeptide of 479 amino acid residues with a predicted molecular mass of 55 kDa. Although discussed more fully below in regard to the tissue distribution, the Mch4 polypeptide is encoded by an approximately 4.0 kb mRNA. This size, together with the presence of an in-frame stop
15 codon 12 bp upstream from the initiator methionine indicates that the cloned Mch4 cDNA (SEQ ID NO:1) contains the full length coding region.

Following identification of and cloning of Mch4, a subsequent search of the GenBank database resulted in the identification of a second novel EST sequence (N42544) with extensive homology to Mch4. Briefly, a homology search of the
20 GenBank database of human expressed sequence tags (ESTs) for sequences similar to Mch4 revealed a 449 bp EST sequence (N42544) with a 64% identity to Mch4. Using PCR primers derived from that EST sequence (Mch5-pr1, GACAGAGCGAGATTCTGT; Mch5-pr2, GCACCATCAATCAGAAGG (SEQ ID NOS:7 and 8, respectfully)) and the vector specific primers T3 and SK-Zap, the full
25 length cDNA corresponding to this gene was amplified by PCR from the Jurkat cDNA library and cloned in KS-vector. To perform this amplification, the Mch5-pr1 and the T3 vector primers were used for the primary PCR amplification step to amplify 5' sequences of Mch5 while the Mch5-pr2 and the SK-Zap vector specific primer was used for the secondary amplification step. The full length cDNA was sequenced and its gene
30 product was named Mch5 (SEQ ID NO:3).

The Mch5 cDNA encodes an about 496 amino acid protein (SEQ ID NO:4) with the highest degree of homology to Mch4 compared to other family members. Excluding the prodomain, the overall sequence identity between Mch4 and Mch5 is about 46%. A comparison of the amino acid sequence identities between

5   FADD and the FADD-like domain within Mch4 and Mch5 is shown in Figure 3A while a multiple amino acid sequence alignment of all known ASCPs is shown in Figure 3B. Although the sequence comparisons of Mch4 and Mch5 are discussed further below. These results indicate that Mch4 and Mch5 are in fact distinct ASCPs and not variants of a single gene product.

10   The identification and sequence analysis of the novel apoptotic proteases described herein has now revealed that both Mch4 and Mch5 belong to the Ced-3-like subfamily of ASCPs. Briefly, previously identified ASCPs can be divided phylogentically into three subfamilies. The Ced-3-like ASCP subfamily includes Ced-3 (SEQ ID NOs: 37-41), CPP32 (SEQ ID NOs: 32-36), Mch2 (SEQ ID NOs: 27-31), and

15   Mch3 (SEQ ID NOs: 22-26). The ICE-like ASCP subfamily includes ICE (SEQ ID NOs: 42-46), TX (ICH2, ICErel-II, Mihl; SEQ ID NO: 47-51) and ICErelIII (SEQ ID NOs: 52-56). The NEDD-like subfamily include ICH-1 and its mouse counterpart NEDD2 (SEQ ID NOs: 57-61). Sequence alignment of Mch4 and Mch5 with these known ASCPs is shown in Figure 3B and reveals both of these new ASCPs belong to

20   the Ced-3-like subfamily of ASCPs.

          Both Mch4 and Mch5 contain N-terminal FADD-like death effector domains. The N-terminal death effector domain of FADD (Hsu et al., Cell, 84:299-308 (1996)) can bind one of the two FADD-like domains in either Mch4 or Mch5 for activation and recruitment to Fas-apoptotic pathway. Activation of Mch4 or Mch5 by

25   FADD can, for example, lead to activation of downstream proteases such as CPP32 and Mch3. Shown in Figure 3A is multiple amino acid sequence alignment of FADD and each of the FADD-like domains within Mch4 (Mch4A and Mch4B) and within Mch5 (Mch5A and Mch5B).

          Shown in Figure 3B is a multiple amino acid sequence alignment of

30   relatively conserved regions within the ASCPs. These regions include, for example, (1)

the active site pentapeptide QACRG (SEQ ID NO:10), (2) the substrate binding residues P1-P4 and (3) the putative processing sites between the small and large subunits. Relevant sequence comparisons for each of these regions as well as other worthy distinctions is discussed more fully below.

5   For example, in the region that does not contain the propeptide domain, Mch4 and Mch5 are equally related to Ced-3 (SEQ ID NO:9) exhibiting an overall amino acid identity of 32% and sequence similarity of 54%. Comparing with the other human Ced-3-like subfamily members, Mch4 is more related to Mch2 and Mch3 (SEQ ID NO:11 and 12, respectfully) with a 38-40% sequence identity and a 56-58%

10   similarity than it is to CPP32 (SEQ ID NOs: 32-36). The latter comparison revealing a 35% amino acid identity and a 57% amino acid sequence similarity. On the other hand, Mch5 is equally related to CPP32, Mch2 and Mch3 with a 39-40% amino acid sequence identity and a 60-62% sequence similarity.

Comparison of Mch4 and Mch5 reveals a significant degree of homology

15   with an overall sequence identity of 52% and similarity of 67% at the primary amino acid level excluding the propeptide domain. As shown in Figure 3B, the homology between the two proteins is highest within the small subunit region. A similar relationship was observed with other family members such as CPP32/Mch3 and ICE/TX. These sequence similarities indicate that Mch4 and Mch5 similarly likely

20   interact with each other as do their related family members CPP32 and Mch3 (Fernandes-Alnemri et al., Cancer Res. 55:6045-6052 (1995b)). For example, Mch4 and Mch5 likely heterodimerize with each other to form functional protease heterocomplexes as do CPP32 and Mch3.

Sequence alignment also revealed that, although distinct, Mch4 and

25   Mch5 are structurally similar to other known ASCPs. The active enzymes of Mch4 and Mch5 are made of two subunits, derived from precursor proenzymes (Mch4 and Mch5) by cleavage at highly conserved Asp residues (Asp239 in Mch4 and Asp284 in Mch5) located between the two subunits (denoted as D/X in Figure 3B) Consistent with other ASCPs, Mch4 and Mch5 are likely processed further to remove the propeptide domains.

Several aspartate cleavage sites are present in the prodomain region of both Mch4 and Mch5 (Figure 3A).

Regardless of the above similarities, one major difference between Mch4 and Mch5 and other family members is that their active site pentapeptide contains an
5 Arg to Gln non-conservative substitution. The substitution changes the previously conserved peptapeptide sequence from QACRG (SEQ ID NO:11) to QACQG (SEQ ID NO:10). Such a substitution could have major effects on enzyme and substrate specificities. The presence of QACQG (SEQ ID NO:10) instead of QACRG (SEQ ID NO:11) in these two enzymes, suggests that other unknown family members with a
10 similar substitution may exist. This result further increases the complexity of the ASCP family.

Another major difference between Mch4 and Mch5 and other ASCP family members is the inclusion of multiple FADD-like domains at their amino termini. The inclusion of these domains indicates that they can interact with FADD early within
15 the fas mediated apoptotic pathway to regulate programmed cell death. Consequently, FADD may bind the FADD-like domains in Mch4 or Mch5 for activation and recruitment to the fas apoptotic pathway. This recruitment occurs because FADD-like. domains are capable of both homotypic and heterotypic interactions (Bold et al., J. Biol. Chem. 270:7795-7798 (1995); Chinnaiyan et al., Cell 81:505-512 (1995); Hsu et al.,
20 *supra*, 1996).

In regard to specific amino acid residues that have been implicated to play functional roles, the crystal structure of ICE has indicated that the amino acid residues His237, Gly238 and Cys285 are involved in catalysis, while Arg179, Gln283, Arg341 and Ser347 are involved in binding the carboxylate side chain of the substrate
25 P1 aspartate. With the exception of Ser347 in Mch5, all of these other residues are absolutely conserved in all family members. Nevertheless, the Ser to Thr substitution in Mch5 corresponding to Ser347, is a conservative substitution and it is the only one among all the family members (Figure 3B). Another Ser to Thr conservative substitution can also be seen in Mch4 in the region corresponding to Ser236. This
30 residue is one that participates in binding the substrate P2-P4 residues. However, others

residues that might participate in binding the substrate P2-P4 residues are not widely conserved. This result indicates that these other residues likely determine substrate specificity.

5   EXAMPLE II

TISSUE DISTRIBUTION AND CHROMOSOMAL LOCALIZATION OF MCH4

This Example shows the expression pattern of Mch4 as measured by RNA blot analysis and the genetic locus of the Mch4 gene.

The tissue distribution of Mch4 was analyzed by RNA blot analysis of

10   poly A^+ RNA isolated from different human tissues. Briefly, tissue distribution analysis of Mch4 mRNA was performed on RNA blots prepared by Clontech (San Diego, California) containing 2 µg/lane of poly A^+RNA from each tissue of origin. A radioactive Mch4 riboprobe was prepared using Mch4 cDNA as a template for T7 RNA polymerase in the presence of $[\alpha^{32}P]ATP$. The blots were hybridized, washed and then

15   visualized by autoradiography.

The results of the RNA blots revealed that a major 3.7 Kb Mch4 message was detectable in most tissues examined. The lowest expression of Mch4 mRNA was seen in whole brain, kidney, prostate, testis and colon. The size of the Mch4 mRNA is consistent with the length of the cloned Mch4 cDNA (3.6 kb). Other higher molecular

20   weight mRNA species can also be seen in some tissues such as skeletal muscle, for example, and could represent unprocessed Mch4 mRNA or an mRNA of a related family member.

To determine the chromosomal localization of the Mch4 gene, a panel of rodent-human somatic cell hybrids was screened by PCR with Mch4 specific primers.

25   Briefly, A panel of DNAs from rodent-human somatic cell hybrids was screened by PCR with the previously described Mch4 specific primers t96-prl (SEQ ID NO:5) and a second Mch4 specific primer termed t96-pr5 (CGGGAGATCATGTCTCAC, SEQ ID NO:9). These primers were also used to screen by PCR the CEPH A and B YAC libraries.

The results of these searches identified two YAC clones (756A9 and 800G4) which were positive for Mch4. A computer search through the Whitehead Institute and CEPH databases showed that both YACS were part of the WI contigs WC-630 and Wc2.16 and of the CEPH contig at position 2.08 of chromosome 2. Other

5   YACS (741D10, 762C12, 809H8, and 828E8) reported by the databases to overlap with 756A9 and/or 800G4 were tested by PCR for the presence of Mch4 gene sequences. Clones 762C12 and 828E8 were found to be positive for Mch4. This analysis resulted in the assignment of Mch4 to chromosome 2p12-qter. To confirm these mapping results and to obtain a definite physical localization for the Mch4 gene, the non-

10 chimeric YAC 828E8 was used in FISH analysis to probe normal human lymphocyte metaphases. The Mch4 chromosomal localization was narrowed to chromosome 2q33-34 using this latter analysis. This places the Mch4 gene within a 4cM region flanked by the centromeric marker D2S374 and the telomeric marker D2S346 (Chumakov et al., Nature 377(supp.):175-183 (1995)).

15

EXAMPLE III

KINETIC PARAMETERS OF MCH4

This Example characterizes the protease activity and substrate specificity of the ASCP Mch4.

20   The kinetic properties of bacterially expressed recombinant Mch4 were determined using the tetrapeptide substrates DEVD-AMC and YVAD-AMC in a continuous fluorometric assay (Table I). The DEVD-AMC and the YVAD-AMC represent the cleavage sites for the poly(ADP-ribose)polymerase (PARP) and IL-1β P1-P4 substrate tetrapeptides, respectively (Nicholson et al., Nature 376:37-43 (1995)).

25 Briefly, Mch4 cDNA lacking most of the propeptide coding sequence (amino acids 61-346) was subcloned in-frame into the Bam HI/XhoI sites of the bacterial expression vector pGEX-5X-3 (Pharmacia Biotech Inc.). This vector produces Mch4 as a fusion protein with glutathione S-transferase (GST) and was used essentially as described in Fernades-Alnemri et al., supra (1995a). The GST-Mch4 expression vector was

30   constructed and transformed into DH5α bacteria using routine molecular biology

methods known to those skilled in the art. After induction with IPTG, bacterial extracts were prepared from *E. coli* expressing the recombinant fusion proteins. The extracts were adsorbed to glutathione-Sepharose resin, washed several times and then analyzed by SDS-PAGE. The Mch4 preparation contained a protein that migrated as a doublet of

5 approximately 50 kDa (GST-large subunit fusion) and 12kDa (small subunit).

The purified Mch4 GST-fusion protein was then used for further enzymatic analyses. The activity of Mch4 was measured using bacterial lysates prepared with ICE buffer (25 mM HEPES, 1 mM EDTA, 5mM DTT, 0.1% CHAPS, 10% sucrose, pH 7.5) at room temperature (24-25°C). The Ki's were determined from

10 the hydrolysis rate of 50 µM DEVD-AMC following a 30 min preincubation of the enzyme with inhibitors DEVD-CHO and recombinant CrmA protein. Prior to incubation with enzyme, purified CrmA was activated by incubation with 5 mM DTT for 10 min at 37°C.

Table I: Kinetic Parameters of Mch4

15

Parameter	Value
K_m (DEVD-AMC)	130 µM
K_m (YVAD-AMC)	150 µM
K_i (DEVD-CHO)	14nM
K_i (CrmA)	0.75 µM

As shown above in Table I, the K_m values of Mch4 for the two peptide substrates DEVD-AMC and YVAD-AMC are similar. These values contrast with those for CPP32, where the K_M for the YVAD-AMC substrate is >35-fold higher than the K_M

20 for the DEVD-AMC substrate (Fernandes-Alnemri et al. supra (1995b)). These kinetic references are further illustrated by the ratio of Vmax/Km for the DEVD-AMC substrate. Specifically, CPP32 possesses a >500-fold higher specificity for this substrate compared to Mch4 ($V_{max}K_{MCPP32}$=9200 and V_{max}/K_{mMch4}=18). However, similar to CPP32 and Mch3α, Mch4 is potently inhibited by the DEVD-CHO peptide

25 (K_{iMch4}=14 nM) and weekly inhibited by Crm A (K_{iMch4}=0.75 µM) (Fernandes-Alnemri

et al., supra (1995b)). Since DEVD-CHO also blocks cell death, this result further indicates that Mch4 is an ASCP which plays a role in the cell death pathway.

EXAMPLE IV

GRANZYME B ACTIVATES MULTIPLE MEMBERS OF THE
MAMMALIAN CED-3 SUBFAMILY

This Example shows that the cytotoxic T cell protease essential for induction of apoptosis in target cells directly activates ASCP members of the Ced-3 subfamily by cleavage into the large and small protease subunits.

Granzyme B has been shown to cleave CPP32 to generate an ~20 kDa cleavage product presumed to be the large subunit of CPP32 (Darmon et al., Nature 377:446-448 (1995)). This cleavage event has attracted the idea that the granzyme B cleavage occurs at the processing sequence IETD-S between the two subunits of CPP32 (Figure 3B). Sequence comparison of the Mch4 and Mch5 ASCPs described herein has revealed that the potential processing sequences between the two subunits of Mch3 and Mch4 are very similar to that of CPP32 (Figure 3B). These two sequences contain identical P1 residues (CPP32-D175, Mch3-D198, Mch4-D239) and P4 residues (CPP32-I172, Mch3-I195, Mch4-I236) in all three proenzymes and a conserved P3 residue (CPP32-E173, Mch3-Q197, Mch4-E237), suggesting that if the processing site in CPP32 is in fact cleaved by granzyme B, then these other subfamily members may similarly be substrates for cleavage as well.

To determine whether granzyme B can cleave these proenzymes at the proposed processing sites, mutant proenzymes with a P1 substitution mutation converting D to A in CPP32 and Mch3 or a D to G in Mch4 were generated. Briefly, potential aspartate processing sites between the two subunits of these ASCPs were mutated to alanine (CPP32 and Mch3) or glycine (Mch4) by site directed mutagenesis using overlapping PCR mutagenic oligonucleotides. Two internal mutagenic overlapping oligonucleotide primers encoding the D/A or the D/G mutation and two external oligonucleotides encoding the first six N-terminal amino acids and last six C-terminal amino acids, respectively, were used in a PCR reaction with CPP32, Mch3 and

Mch4 cDNAs. Asp9 of proCPP32 was mutated to Ala by PCR using a 5' mutagenic oligonucleotide encoding the D to A mutation and a 3'- primer derived from the 3'-noncoding sequence of CPP32 cDNA. The resulting PCR products were subcloned in pBluscript II KS$^+$ vector under the T7 promoter and their sequence was verified by DNA sequencing.

Wild type and mutated cDNAs were *in vitro* transcribed and translated in the presence of ^{35}S-methionine using Promega coupled transcription/translation TNT kit according to the manufacturer recommendations. Two microliters of the translation reactions were incubated with purified enzymes (100-200 ng) or bacterial lysates expressing recombinant ASCPs in ICE-buffer, in a final volume of 10 μl. The reaction was incubated at 37°C for 1-2 hours and then analyzed by SDS-PAGE and autoradiography.

Following *in vitro* translation, the parental and mutant proenzymes were incubated with granzyme B and then analyzed by SDS-PAGE and autoradiography. As shown in Figure 4A, *in vitro* translation of wild type (lane 1) or Asp175-mutated (lanes 2 and 3) CPP32 proenzymes generated identical pattern of translation products. The major translation products started with Met27 and Met39, respectively. Incubation of these translation products with granzyme B resulted in cleavage of the wild type proCPP32 at Asp175 (lane 5) to generate the two subunits of active CPP32. The small C-terminal subunit migrates as a single ~12 kDa band and the large N-terminal subunit migrates as three bands (~21, ~19 and ~17 kDa).

In regard to the identities of the bands comprising the large subunit, the faint ~21 kDa band is most likely a cleavage product of the full length proCPP32. However, the high intensity of the ~19 kDa band suggests that it is produced from the ~21 kDa band by further processing at the propeptide domain. This indication is supported by the observation that incubation of proCPP32 with granzyme B in the presence of the CPP32 peptide inhibitor DEVD-CHO, generated a major ~21 kDa band which was not further processed to the ~19 kDa band (Figure 4B, lane 8). Further processing of the ~21 kDa band to the ~19 kDa band was only observed in the absence of the peptide inhibitor DEVD-CHO (Figure 4A, lane 5 and Figure 4B, lane 9). This

result indicates that the additional processing of the propeptide domain seen with the wild type proCPP32 is due to the autocatalytic activity of the granzyme B-activated CPP32. No cleavage was observed with the buffer control or the Asp175-mutated CPP32 (Figure 4A, lanes 1 and 3, respectively).

5   In addition there was no cleavage at the propeptide domain of the Asp175-mutated CPP32 (Figure 4A, lane 2 and Figure 4B, lane 3), indicating further support to our earlier conclusion that cleavage of the propeptide is an autocatalytic activity of activated CPP32. Furthermore, the autocatalytic processing within the propeptide domain occurs at Asp9 and not at Asp28. Mutation of Asp9 to Ala inhibited

10   the processing of the ~21 kDa band to the ~19 kDa band in a similar fashion as observed with the DEVD-CHO inhibitor (Figure 4B, lanes 5 and 6). These data indicate that CPP32 is autocatalytically processed at Asp9 after activation to generate a p19 (large subunit) and p12 (small subunit).

Earlier observation that purified human CPP32 was processed at Asp28, could be due to the fact that CPP32 was purified from THP-1 monocyte cytosol after

15   incubation at 37°C for several hours (Nicholson et al., supra (1995)). THP-1 cytosol contains high concentration of ICE and possibly other ICE homologs that might be responsible for the additional processing at Asp28. The ~17 kDa band is a cleavage product of one of the smaller internally translated products, most likely the 29 kDa

20   band.

Similarly, in vitro translation of wild type or Asp198-mutated proMch3 (Figure 5A, lanes 1 and 2, respectfully) generated two major products. These two translation products are a 35-36 kDa product corresponding to the full length proMch3 and a 30 kDa internal translation production most likely starting with Met45. Other

25   internal translation products smaller than 30 kDa can also be seen.

Like proCPP32, incubation of proMch3 with granzyme B generated the two subunits of the active Mch3 enzyme (Figure 5A, lane 6). These subunits can be seen as a ~12 kDa band corresponding to the small C-terminal subunit and two ~20 and ~18 kDa bands corresponding to the large N-terminal subunit. The ~20 kDa band is a

30   product of processing at Asp198 between the two subunits, and at Asp23 in the

propeptide domain. The ~18 kDa band is a cleavage product of the smaller 30 kDa internally translated product. No cleavage products corresponding to the small or large subunits were observed with the buffer control or the Asp198-mutated proMch3 (Figure 5A, lanes 1, 2 and 4, respectively).

5   Unlike CPP32, there was a 33 kDa cleavage product in the Asp198-mutated proMch3 (lane 4). This product is a result of granzyme B cleavage in the propeptide domain of proMch3, indicating that granzyme B can process proMch3 to active Mch3 without the requirement of an additional activity to remove the propeptide domain. Nevertheless, we have shown recently that CPP32 can also cleave the

10   propeptide domain of proMch3 very efficiently (Fernandes-Alnemri et al., supra (1995b)). Consequently, activation of CPP32 in vivo by granzyme B would result in further processing of both CPP32 and its closely related homolog Mch3.

      Two truncated Mch4 lacking the N-terminal FADD-like domains were used to analyze cleavage of Mch4 by granzyme B. Mch4-M134 starts with amino acid

15   residue M134 and proMch5-M235 starts with amino acid residue M235. In vitro translation of Mch4-M134 or Asp239-mutated Mch4-M134 (Figure 5B, lanes 4 and 5, respectfully) generated two major products. These two translation products are observed as a 39 kDa product corresponding to the full length Mch4 and a 27 kDa internal translation product. The internally translated product starts with Met235. This

20   was confirmed by deletion of the cDNA sequence encoding the first 101 amino acids and allowing the translation to proceed from Met102. This deletion produced a truncated Mch4 protein that was similar in size to the internally translated ~27 kDa Mch4 protein (Figure 5B, lane 1).

      Granzyme B cleaved the truncated Mch4-M235 to generate 15-16 kDa

25   and 12 kDa bands (Figure 5B, lane 3). On the other hand, Granzyme B cleaved the full length Mch4-M134 to generate ~27 kDa band (large subunit) and 12 kDa band (small subunit) (lane 8). However, because of the presence of the internally translated 27 kDa protein together with the full length Mch4, a 15-16 kDa band was also produced after incubation with granzyme B (lane 8). Like CPP32 and Mch3, the Asp239-mutated

Mch4-M134 was not cleaved by granzyme B (lane 6), and there was no cleavage in the buffer control (lanes 1 and 4).

These data show that granzyme B not only activates pro-CPP32, but also the related ASCPs Mch3 and Mch4 by cleavage at the IETD-S, IQAD-A and IEAD-A

5   putative processing sequences, respectively. Cleavage at these sites generates the two subunits that form the active enzyme complex of these proteases.

EXAMPLE V

MCH4 IS UPSTREAM OF CPP32 AND MCH3 IN THE ASCP CASCADE

10   This Example shows that Mch4 is capable of activating both proCPP32 and proMch3 while remaining resistant to cleavage from its proenzyme state when incubated in the presence of activated forms of either CPP32 or Mch3.

Evidence suggests that ASCPs are involved in a cascade of activation events which leads to the final cell death signal. To determine whether such a cascade

15   exists within the Ced-3 subfamily of ASCPs occurs, the activation of CPP32 and its closely related homolog Mch3 by another subfamily member such as proMch4 was assessed. Activation was determined by incubating purified recombinant Mch4 with proCPP32 and proMch3.

Analysis of the cleavage products showed that proMch4 processed

20   proCPP32 and proMch3 and generated cleavage products identical to those produced by granzyme B (Figure 4A, lane 4 and Figure 5A, lane 5). Mch4 was unable to process the Asp to Ala mutated proCPP32 and proMch3 (Figure 4A, lane 3 and Figure 5A, lane 3). However, like granzyme B, Mch4 was able to cleave the propeptide of Mch3 to generate a 33 kDa band (Figure 5A, lane 3). Although Mch4 was able to cleave

25   proMch4, its activity towards its proenzyme was significantly lower than that towards proCPP32 and proMch3 (Figure 5B, lane 7). In addition there was no significant cleavage of proMch4 when incubated with recombinant CPP32 or Mch3 enzymes (Figure 6). The activity of several other ASCPs such as ICE, TX and Mch2, were also tested but none of these enzymes were able to efficiently process proMch4. These data

30   indicate that Mch4 is upstream of CPP32 and Mch3 in the apoptotic protease cascade.

The above results indicating that proMch4, Mch3 and CPP32 play a role in a protease cascade are further supported by the unique features exhibited by these and related ASCPs. Specifically, ASCPs have two unique features that distinguish them from other proteases. First, they all cleave their substrates after Asp residues, and their

5   activation requires cleavage after Asp residues located in highly conserved processing sites between their large and small subunits. The ability to cleave after Asp residues is only shared with granzyme B, a serine protease that does not however require cleavage after Asp residues for its activation. In addition to these features, both Mch4 and Mch5 contain N-terminal FADD-like death effector domains. The N-terminal death effector

10 domain of FADD (Hsu et al., Cell, 84:299-308 (1996)) can bind one of the two FADD-like domains in either proMch4 or proMch5 for activation and recruitment to Fas-apoptotic pathway. Activation of proMch4 or proMch5 by FADD can, for example, lead to activation of downstream proteases such as CPP32 and Mch3.

The above features indicate that ASCPs interact with and activate each

15 other in a protease cascade fashion as well as acting as substrates for granzyme B. In addition, because multiple ASCP family members coexist in one cell type the ability of one family member to activate several other family members and *vice versa* results in multiple protease cascades and the generation of multiple apoptotic pathways. Evidence for the existence of multiple apoptotic pathways is corroborated from studies with mice

20 deficient in ICE or Bcl2. For example, thymocytes from ICE deficient mice remain sensitive to glucocorticoid- and ionizing radiation-induced apoptosis, but become resistant to antiFas-induced apoptosis (Kuida et al., Science 267:2000-2003 (1995)). On the other hand, T-cells from bcl2 deficient mice become more sensitive to glucocortiocoid- and ionizing radiation-induced apoptosis, but less sensitive to

25 antiCD3-induced apoptosis.

As shown in Figure 7, the above results indicate the existence of multiple protease cascades that can be activated by different apoptotic stimuli. For example, one of these cascades involves proMch4 acting upstream of CPP32, Mch2 and Mch3. Once proMch4 is activated by certain apoptotic stimuli, it can process and activate the

30 proenzymes of Mch3 and CPP32 as shown above. These two ASCPs are likely

responsible for PARP cleavage in apoptosis. Active CPP32 can in turn activate proMch2, the only ASCP that can cleave lamin. Because CPP32, Mch3 and proMch4 are poorly inhibited by CrmA (see Table I), the above cascade would not be affected in an ICE-knockout mice, or inhibited by the ICE inhibitor Crm A. Therefore, it is likely

5   that glucocortiocoid- and radiation-induced apoptosis occur through this cascade.

        In an alternative ICE or an ICE-like pathway, activation of ICE or an ICE-like ASCP like TX by an apoptotic stimulus or an upstream ASCP results in CPP32, Mch2 and Mch3 activation (Figure 7). This result is because TX can activate ICE (Faucheu et al., The EMBO J. 14:1914-1922 (1995)) and ICE can activate

10   proCPP32 (Tewari et al., Cell 81:801-809 (1995)). Furthermore, Mch5 can process proCPP32 and proTX. This ICE-like pathway likely operates in the Fas-apoptotic pathway, since ICE knockout or CrmA abrogate this pathway in some cell types. Also, during Fas-induced apoptosis an ICE-like activity precedes CPP32-like activity (Enari. et al., Nature 380:723-726 (1996)). Consequently, FADD likely binds to FADD-like

15   domain in proMch5 or proMch4 for activation and recruitment to Fas-apoptotic pathway. This conclusion is because these domains are capable of both homotypic and heterotypic interactions. Once bound to FADD, proMch5 can undergo autocatalytic processing to the mature enzymes. In this alternative, mature proMch5 could also activate proCPP32 directly, or indirectly by activating proTX. Mature CPP32 would in

20   turn activate the lamin cleaving enzyme Mch2.

        The most N-terminal or first domain in both proMch4 and proMch5 (Mch4A; Figure 3A) are highly related to FADD and likely act as activators of proMch4 and proMch5 by binding the second C-terminal FADD-like (interacting) domain. It is likely that either proMch4 or proMch5 mediates Fas apoptosis by interacting with

25   FADD. However, because they have two N-terminal FADD domains, these polypeptides can be involved in other forms of apoptosis. For example, proMch4 or proMch5 can be repressed under normal conditions by a repressor that sits on its N-terminal FADD domain. Alterations in cellular conditions could release the repressor allowing the N-terminal domain to interact with the second C-terminal FADD-

interacting domain leading to activation of proMch4 or proMch5 and consequently activation of downstream proteases such as CPP32, Mch3 and Mch2.

In yet another distinct apoptotic protease cascade an exogenous protease is used to activate multiple endogenous ASCPs. This is the granzyme B-cascade which is used by cytotoxic T-lymphocytes to kill their target cells (see Figure 7). With the understanding of these multiple cascades and their regulatory activation events, it is now possible to target these pathways either alone or in combination for the therapeutic treatment of human diseases.

EXAMPLE VI

PROMCH4 EXHIBITS CELL DEATH ACTIVITY

This Example shows the expression of proMch4 and induction of apoptosis in cultured cells.

To determine if proMch4 exhibits cell death activity, the induction of early apoptosis in Sf9 baculovirus cells was assessed. Briefly, Sf9 cells were infected with recombinant baculoviruses encoding full length proMch4 or full length CPP32 as a standard (Fernandes-Alnemri et al., J. Biol. Chem. 269:30761-30764 (1994)). Cells were then examined microscopically for morphological signs of apoptosis such as blebbing of the cytoplasmic membrane, condensation of nuclear chromatin and release of small apoptotic bodies. In addition the genomic DNA was examined for internucleosomal DNA cleavage.

For the construction of transfer vectors and recombinant baculoviruses, the full length proMch4 in pBluescript KS+ was excised with Bam HI and EcoRI and subcloned into a Bam HI/EcoRI cut pVL1393 vector (Invitrogen, San Diego, California) to generate the pVL-proMch4 transfer vector. The pVL-CPP32 transfer vector was made as described previously (Fernandes-Alnemri et al., supra (1994)). The recombinant transfer vectors were then used to generate recombinant Baculoviruses as previously described (Summers et al., "Manual of Methods for Baculovirus Vectors and Insert Culture Procedures," Texas Experimental Station Bulletin No. 1555 (Texas A&M

University, College Station, Texas (1987); and Alnemri et al., J. Biol. Chem. 266:3925-3936 (1991)).

For-the induction of apoptosis in Sf9 cells by proMch4 and CPP32 cells were infected with recombinant baculoviruses AcNPV-proMch4 or AcNPV-CPP32.

5 Apoptosis was measured microscopically by counting cells with the appropriate morphology (blebbing, nuclear condensation). Alternatively, internucleosomal DNA cleavage is assessed as a characteristic marker. Briefly, total cellular DNA is isolated at 42 h postinfection from either control Sf9 cells or Sf9 cells infected with AcNPV-proMch4 or AcNPV-CPP32 baculoviruses (Alnemri et al. supra (1995)). The DNA

10 samples were analyzed by electrophoresis in a 1.8% agarose gel containing ethidium bromide.

Expression of full length proMch4 in Sf9 cells caused a significant percentage of the cells to undergo apoptosis by about 48 h postinfection which is also manifested by induction of internucleosomal DNA cleavage. These results are

15 consistent with proMch4 being a cell death protease since AcNPV-CPP32 yielded similar results.

EXAMPLE VII

THE MCH5 FADD HOMOLOGY DOMAIN B INDUCES APOPTOSIS.

20 To determine if the expression of the FADD-like domain B of proMch5 (Mch5B) can induce apoptosis, it was cloned into a mammalian expression vector and transfected into the MCF7 human breast carcinoma cell line.

After transfection (36 hours), the percentage of transfected cells that were apoptotic was counted. Figure 8 shows that in cells transfected with the control

25 plasmid pcDNA3 about 50% of the cells were apoptotic. This result is likely due to the induction of apoptosis by the lipofection reagent used for DNA transfection. In contrast, about 80% of the cells transfected with Mch5B were apoptotic (Figure 8). Thus, heterologous expression of Mch5 FADD-like domain B induces apoptosis in these cells.

The induction of apoptosis by Mch5B indicates that the mechanism by which Mch5B induces apoptosis is similar to the way in which the homologous domain in FADD (the FADD death effector domain) induces apoptosis when expressed by transfection. This mechanism involves binding of the Mch5 FADD-like domain to 5 either the proMch4 or proMch5 pro-domains, binding induces activation of the proMch4 or proMch5 proteases and induction of apoptosis.

Briefly, Mch5B was subcloned into the mammalian expression vector pcDNA3. The Mch5 cDNA in the vector pBluescript KS was used as a template for PCR amplification of the Mch5 FADD B domain using the following primers: 5' 10 primer: CCTACAGGATCCACTTCTGCCGCATGAGC; 3' primer: ACTCCTCCCCTTTGCTGAATTCTTAATAGTCGT. The PCR product was cut with BamHI and EcoRI and ligated into BamHI/EcoRI cut pcDNA3 to produce the Mch5/Fadd B/pcDNA3 (MFp) vector. MFp DNA was transduced into DH5α bacteria and DNA was purified. For transfection, MFp or pcDNA3 (1.8 µg) were mixed with 15 the liofectin reagent (GIBCO Life Technology) and 0.2 µg of plasmid pCMC-SPORT-βgal (GIBCOBRL Catalogue #10586-014) and applied to 50% confluent cultures of MCF7 cells for eight hours at 37°C. The cells were then washed and growth media added. After 36 hours cells were fixed in 10% para-formaldehyde and β galactocidase expression visualized by incubating cells with X-gal substrate solution.

20 Although the invention has been described with reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention. It should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

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